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Bacterial Survival in Snow Made from Wastewater

Louise V. Parker, Melinda L. Yushak, C. James Martel,
and Charles M. Reynolds

July 2000

Abstract: This study examined the effects of a patented wastewater treatment process that makes snow from secondary wastewater, and the subsequent freeze-thaw cycling processes that occur in a snow column, on bacterial survival. Coliform bacteria were observed to be the most adversely affected by snowmaking, with more than a 3-log reduction in

the total coliform counts and more than a 2-log reduction in the fecal coliform counts. Other species of bacteria were less affected by snowmaking, especially the gram-positive, fecal streptococci. Many species of bacteria also survived the multiple freeze-thaw cycles in the snow column and replicated during melting.

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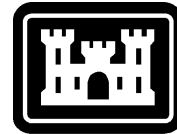
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OFFICE OF THE CHIEF OF ENGINEERS

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PREFACE

This report was prepared by Louise V. Parker, Research Physical Scientist, Applied Research Division, and by Melinda L. Yushak, Engineering Technician, C. James Martel, Environmental Engineer, and Charles M. Reynolds, Research Physical Scientist, Geochemical Sciences Division, U.S. Army Cold Regions Research and Engineering Laboratory (CRREL), Engineer Research and Development Center (ERDC), Hanover, New Hampshire.

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Bacterial Survival in Snow Made from Wastewater

LOUISE V. PARKER, MELINDA L. YUSHAK, C. JAMES MARTEL,
AND CHARLES M. REYNOLDS

INTRODUCTION

Wastewater is the used water supply of a community, and thus is a dilute solution of fecal matter and wastes. One of the primary purposes of wastewater treatment is to remove constituents that can reduce the quality of receiving waters. This treatment includes removing any substances that would increase color, odor, or clarity, or decrease dissolved oxygen (DO) levels. This includes removing any nutrients that can cause growth of algae and removing any health hazards, such as pathogenic microorganisms or toxic contaminants. Typically, the pathogenic microorganisms that are found in wastewater cause gastrointestinal illnesses characterized by diarrhea and abdominal cramps, which may be accompanied by vomiting and fever. Microorganisms known to cause illness include bacteria, viruses, protozoan cysts, and the eggs (ova) of helminths (parasitic worms). Bacteria of concern include some species of *Shigella*, *Salmonella*, *Leptospira*, and *Vibrio*, and strains of *Escherichia coli* (*E. coli*). Viruses of concern include enteroviruses (including polio, Coxsackie, and infectious hepatitis [type A] viruses), reoviruses, adenoviruses, rotaviruses, and Norwalk-type viruses. Pathogenic protozoa include *Giardia lamblia*, *Cryptosporidium*, *Balantidium coli*, and *Entamoeba histolytica*. Helminths of concern include *Ascaris lumbricoides* (roundworm), *Enterobius vermicularis* (pinworm), and *Trichuris trichiura* (whipworm).

Primary wastewater treatment removes floating and settleable solids by using physical operations such as screening and sedimentation. Secondary treatment removes most organic matter and suspended solids by using biological and chemical processes such as activated sludge, fixed film reactors, lagoons, and sedimentation. Tertiary treatment removes other remaining constituents, including nitrogen (N) and phosphorus (P), through treatment processes such as chemical floccu-

lation, sedimentation followed by filtration and activated carbon, reverse osmosis, and ion exchange.

Delta Engineering (Ottawa, Canada) has developed a patented process, called Snowfluent, that uses secondary wastewater as a water source for snowmaking. During the winter, the wastewater is stored as snow. In the spring, the meltwater discharges first to the unfrozen ground until it becomes saturated. The remaining meltwater is run off and discharges to surface waters. The reported benefits of this treatment process include a high level of treatment; ability to function in the cold, where other processes either fail or are less effective; elimination of the need for new treatment lagoons for wastewater storage in cold weather; elimination of the need for chemical flocculation of phosphorus; elimination of bacteria without requiring disinfectants; a low operating cost; and possible use for revenue-generating agricultural purposes.

According to the manufacturer, most of the contaminants are deposited with snow except for gases such as ammonia and carbon dioxide, which are reduced during snowmaking. The initial meltwater, which contains most of the contaminants, then percolates into the soil surface where the contaminants are adsorbed. These adsorbed nutrients are removed in the warmer months by plant uptake and bacterial degradation. Later meltwater is reported to be relatively uncontaminated and can be allowed to discharge into surface water.

The process by which impurities are concentrated in a snowpack has been explained by Colbeck (1981) as follows: exclusion of impurities occurs after snow deposition during the process of grain coarsening and freeze-thaw cycles that the snowpack undergoes. This concentrates a major fraction of the impurities present in the snowpack onto the outer surface of snow crystals and into interstitial water. From there the impuri-

ties are readily removed by a “wetting front” moving through the snowpack.

We found that relatively little has been published in peer-reviewed journals on the ability of this process to remove organic and inorganic contaminants. Most of the publications that present data (Zapf-Gilje et al. 1986, Rabinowitz et al. 1988) are based on the thesis work of Zapf-Gilje (1985). Zapf-Gilje (1985) reported that, on average, 86% of the N and 65% of the P were concentrated in the initial 20% of the snowmelt. He attributed the lesser degree of concentration of P to its high affinity for particulate matter in the snow. For some metals, such as iron and manganese, there was no evidence of any concentration (Rabinowitz et al. 1988). Passage through a soil column is a key element in this treatment process (Rabinowitz et al. 1988).

Delta Engineering describes the removal of biological contaminants as follows. During snowmaking, “the wastewater is atomized into small droplets, which freeze rapidly. The mechanical forces inherent in the freezing process, combined with the rapid freezing and expansions of the droplets, cause rupture of the outer membrane of the microorganisms, thereby killing them. A relatively few surviving pathogens held within the snowpack are either too severely damaged to reproduce, or are ultimately eliminated through exposure to the sun’s ultraviolet rays. Studies show that Snowfluent™ disinfects wastewater more effectively than any traditional treatment methods such as chlorination, ozone, or UV radiation.”

Again we found relatively little information published in the peer-reviewed literature on the effectiveness of this type of process on biological contaminants. The literature indicates that there is partial destruction of some species of bacteria, especially coliforms, but that bacteria are not completely destroyed by this process. Rabinowitz et al. (1988) reported that snowmaking reduced total coliform and fecal coliform concentrations by 50%. In Zapf-Gilje’s thesis (1985), he reported work described in an unpublished draft report by the Ontario Ministry of Environment (1982) that revealed the following. There was more than a 99%, or 2-log reduction, in total and fecal coliforms during snow production, with further reduction of total coliforms in the snowpack. Other strains of bacteria had higher survival rates during snowmaking, but no details were given. Destruction was attributed to the freezing process because microorganisms in atomized, unfrozen wastewater were not killed while those in atomized, frozen wastewater were. Unfortunately, there was no description of the test methods that were used in this unpublished study. Furthermore, we could find no information on the survival of any other types of microorganisms of health concern, such as helminths, viruses, and protozoa.

We wondered whether the outer membrane of microorganisms would be destroyed by this process given that gram-positive bacteria have an inner cell membrane but no outer membrane and that other microorganisms, such as viruses, consist only of a nucleic acid (either DNA or RNA) contained in a protein or lipoprotein coat and thus do not have a cell membrane. Because of differences in the cell wall composition and membrane differences between gram-positive and gram-negative bacteria, we wondered whether gram-positive bacteria would be more resistant to the effects of this treatment. We also wondered whether the repeated freeze-thaw cycling would have any effect on bacterial survival in these systems, as reportedly found by the Ontario Ministry of the Environment in its unpublished report.

To answer these questions, we conducted a literature review on the effects of freezing and thawing on bacteria and conducted studies to determine the effect of this process on bacteria.

LITERATURE REVIEW

Effects of chilling, freezing, low-temperature storage, and warming on bacteria

Bacteria can be injured or die as a result of cold shock, freezing, storage at low or subzero temperatures, and subsequent warming. Cold shock is caused by sudden chilling without freezing. Studies have shown that cold shock can damage the cytoplasmic (inner) membrane and DNA of bacteria and can damage the outer membrane of gram-negative bacteria (MacLeod and Calcott 1976, Mackey 1984). Freezing and thawing has been shown to damage the cytoplasmic membrane, cell wall, and DNA (MacLeod and Calcott 1976, Mackey 1984, Ray 1989). When the cytoplasmic membrane is damaged, low molecular weight materials (such as potassium and magnesium cations [K^+ , Mg^{2+}], inorganic phosphate, and amino acids) are lost from the cell, and there is an increased penetrability of small molecular weight compounds, such as toxic metals, into the cell (MacLeod and Calcott 1976). Researchers have attributed death and injury to one or both of these processes. However, depending upon the species and the surrounding medium, many of the cells injured by these processes can self-repair.

Cold shock

Both gram-positive and gram-negative bacteria have been shown to be affected by cold shock (MacLeod and Calcott 1976). Factors that affect the sensitivity of cells to cold shock include age (cold shock usually occurs in cells harvested in the exponential growth but not stationary phase), composition of medium in which

cells are chilled (divalent cations such as magnesium, calcium, and manganese [Mg^{2+} , Ca^{2+} , Mn^{2+}] substantially protect against the effect of chilling and have been shown to mediate recovery), cell number (loss of viability is greater the smaller the cell population), rate of cooling, and temperature range over which cooling takes place. During rapid cooling, permeability changes in the cell membrane are caused by a phase transition in the membrane lipids from a liquid crystalline to a gel state (MacLeod and Calcott 1976). Slow cooling allows a lateral phase separation of the lipids and proteins of the membrane, whereas rapid cooling “fixes” these components in a random, disordered state, resulting in membrane leakiness (Mackey 1984).

Freeze–thaw damage

Both the rates of cooling and warming affect survival of cells that have been frozen and thawed. Different cooling and warming rates produce different kinds of damage (MacLeod and Calcott 1976). Damage varies depending on the chemical composition of the freezing medium, especially the presence of NaCl (MacLeod and Calcott 1976). The type and strain of organism, its phase of growth when frozen, and the temperature and duration of frozen storage are also important factors (Mackey 1984). The initial number of bacteria can also affect survival, with high concentrations having a protective effect (Mazur 1966). Resistance of bacteria to freezing varies widely; cell shape and differences in membrane fatty acids and proteins have been found to affect cryosensitivity (Mackey 1984).

Most cell types, whether procaryotes or eukaryotes, have an optimum cooling rate for survival that varies, depending on the water permeability of the membrane and on the surface-to-volume ratio of the cell (Mackey 1984). For many bacterial species, maximum survival occurs at cooling rates between 6 and 11°C per min (Mazur 1966, MacLeod and Calcott 1976, Mackey 1984). Mazur (1966) proposed that, at slow cooling rates, ice crystals form extracellularly, thus concentrating the solutes in the extracellular solution, thereby causing the cell to dehydrate. Solute concentrations inside and outside the cell then reach levels that can cause denaturation of proteins and breakdown of membranes. At more rapid cooling rates (above this optimum), the temperature is reduced at a faster rate than water can flow through cell membrane. This results in the ice nucleation in intracellular water. At very rapid rates of cooling ($>100^\circ\text{C}/\text{min}$), ice crystal growth is retarded or prevented and survival again is greater. However, very small ice crystals may grow and cause damage if these cells are warmed slowly. Hence survival of ultrarapid cooling is dependent on warming

rate, with rapid warming offering the best chance for survival (Mackey 1984).

We would anticipate that the cooling rate microorganisms would encounter during snowmaking would be very rapid ($>100^\circ\text{C}/\text{min}$)* and thus the effect of freezing at this rate would be less than at slower rates. However, we also anticipate that the warming and cooling rates in the snowpack would be relatively low. This slow freeze–thaw cycling may have more of an effect than the initial freezing process.

Storage death and susceptibility of various bacterial species

Several studies have shown that in addition to the death of cells on initial freezing, there is usually further death during frozen storage. Usually, death occurs rapidly in the early stages followed by a slowing of the rate until, in the later stages, numbers remain almost constant, with greater survival at lower temperatures (Mackey 1984). According to Mazur (1966), death rates are low or zero when storage is at temperatures of -70°C or below, while temperatures between -60°C and 0°C decrease the survival of most species with time. The rate of the decrease in survival depends on the species, the storage temperature, the nature of the freezing medium, and in some cases the cell concentration (Mazur 1966, MacLeod and Calcott 1976). Death is presumed to be mainly due to continued exposure to concentrated solutes (Mackey 1984).

According to Mackey (1984), bacteria vary widely in their response to frozen storage. They found that fecal streptococci and *Staphylococcus aureus* survived well under most conditions, whereas *Vibrio parahaemolyticus*, *Yersinia enterocolitica*, *Campylobacter jejuni*, and vegetative cells of *Clostridium perfringens* declined in numbers by as much as 10^2 to 10^5 within a few weeks at -20°C , and other organisms such as *Salmonella* species and *E. coli* are of intermediate resistance, with their survival highly dependent upon the composition of the frozen medium.

McCarron (1965) studied the survival of six bacterial species in ice at subfreezing temperatures (-2°C , -20°C). Bacteria included three gram-negative rods (*E. coli*, *Aerobacter aerogenes*, *Serratia marcescens*), two gram-positive cocci (*Micrococcus roseus* and *Sarcina lutea*), and one gram-positive, sporeforming rod (*Bacillus subtilis*). McCarron found that more than 90% of the bacteria were inactivated in the first two days but that the remaining cells persisted for several months. *M. roseus* (one of the gram-positive cocci) and the

*Personal communication, Scott Barthold, Sno.matic Controls and Engineering, Inc., Lebanon, New Hampshire, 1999.

spores of *B. subtilis* were most resistant to freezing. *E. coli* succumbed more rapidly than the other organisms during storage.

Kraft (1992) reported that although considerable strain variation exists, vegetative cells of cocci are resistant to freezing and frozen storage, and gram-negative bacteria are less resistant in general than gram-positive bacteria. Spores are very resistant to freezing. With the exception of the gram-positive *Cl. perfringens*, Mackey's (1984) data also appear to support the claim that gram-positive cells are more resistant to freezing. Organisms in logarithmic growth phase are not as resistant as those in stationary phase.

Effects on other microbial pathogens

Although there is a great deal of information on the effect of chilling, freezing, and rewarming (thawing) on bacterial survival, we have not been able to find much information on the effect of these processes on other types of pathogenic, sewage microorganisms, such as helminth eggs, protozoa, or viruses. A study by Sanin et al. (1994) did compare survival of several microorganisms in frozen sludge (Table 1). They found that fecal streptococci and *Ascaris* eggs (parasitic worms) were the most resistant to the effects of freezing, that bacteriophage and polio virus were less susceptible than fecal coliforms, and that the protozoan oocysts were completely destroyed by freezing (>8-log reduction).

With respect to other water treatment processes, Ridgway (1984) reported that in water, the degree of resistance to inactivation of various types of microorganisms was vegetative bacteria < viruses < bacterial spores and protozoa.

MATERIALS AND METHODS

General information

Snow made from secondary wastewater at a treatment facility in Carrabassett Valley, Maine, was used in these studies. To determine the fate of bacteria as a result of spraying, wastewater was collected after it entered the spray system, and freshly fallen manufactured snow was collected. Because this treatment facility operates only when it is able to make snow, we were unable to conduct any tests that would allow us to determine whether cell losses that are associated with spraying were due to freezing or to the rapid change in pressure at the spray nozzle.

Snow columns, which were placed in a temperature-controlled environment or outdoors, were used to determine the impact of overwintering and spring melt on bacteria. For these columns, the freshly fallen, manufactured snow was collected and placed in plastic storage bags, which were then placed in insulated coolers for transport back to our laboratory. The harvested snow was stored at -10°C (15°F) until it was used to build the snow columns.

The snow columns were placed either outdoors or in a low-temperature incubator that was cycled from temperatures from -8°C to $+14^{\circ}\text{C}$. The snow columns were cycled so that the snow column would undergo numerous freeze-thaw cycles. There was no set pattern to the cycling except that daytime temperatures tended to be warmer than nighttime temperatures. The pattern of warming and cooling was deliberately erratic to simulate a typical New England winter. Meltwater was sampled to determine the fate of bacteria during

Table 1. Reduction of pathogenic and indicator microorganisms in two different sludges by freeze-thaw conditioning. (From Sanin et al. 1994.)

	Overall log reduction	
	Aerobically digested sludge	Anaerobically digested sludge
Fecal coliforms	1.90	1.10
Fecal streptococci	0.21	0.20
<i>Salmonella</i>	0.54	0.74
Viral Plaque Forming Units	0.80	0.85
Poliovirus	1.08	1.47
Helminth ova ¹	-0.06	-0.03
Protozoa oocysts ²	>8.00	>8.00

¹*Ascaris*

²*Cryptosporidium parvum*

the “winter.” The meltwater fractions were drawn off into sterile glass dilution bottles. Each bottle was completely filled. In some instances, partially filled bottles were stored briefly in the refrigerator until enough leachate could be collected to fill the bottle.

Snow column construction

Columns were made of 14.9-cm-internal-diameter (i.d.) PVC pipe and were 101.6 cm tall. A 15.9-cm-i.d. end cap was fitted on the bottom end. The end cap was not solvent-bonded but the upper (outside) rim of the end cap was sealed with medium-set PVC cement. So that sample could be drawn from the bottom of the column, a small (~2.5-cm) hole was drilled through the lower end of the PVC pipe and end cap. A plastic barbed fitting was placed in the hole and tightened until it did not leak. Tygon tubing (0.95-cm i.d.) was attached to the fitting and secured to the fitting with a small pipe clamp. A C-type tubing clamp was placed on the other end of the tubing and was left in the closed position except when samples were drawn off. Samples were collected only when there was flow from the tubing; no suction was applied. The sides of the columns were wrapped with a layer of 2.2-cm Armaflex foam insulation to promote warming from the top and bottom of the snow column, rather than the sides, and thus simulate what would happen in the snowpack. Prior to starting an experiment, the inside of the column and tubing were washed with a detergent solution, rinsed with copious amounts of tap water, and then rinsed three times with distilled water.

As snow was packed in the PVC columns, snow samples were taken to determine the initial level of contamination in the snow at the start of the experiment.

Test procedures for snowmaking study

Wastewater was collected after leaving the lagoon and prior to entering the snow guns in 110-mL sterile, polypropylene containers with snap lids. The freshly fallen manufactured snow was collected and placed in the same type of containers. All the containers were placed in insulated coolers containing bags of snow and transported back to our laboratory for analyses later that afternoon and evening. The snow samples were melted at room temperature prior to analyses. Samples were collected from approximately 7:45 a.m. to 9:45 a.m. The high temperature for that day was -10.6°C (13°F) and the low was -19.4°C (-3°F).

Test procedures for snow column studies

First indoor study

In this study, the temperature of the incubator was cycled from well below freezing to slightly below freez-

ing eight times ($\sim -7^{\circ}\text{C}$ to -2°C), from freezing to thawing temperatures 12 times ($\sim -1^{\circ}\text{C}$ to 5°C), from thawing temperatures to even warmer temperatures four times ($\sim -3^{\circ}\text{C}$ to 10°C), and then remained warm on the last day (9°C to 14°C). The complete cycling schedule is given in Table A1.

We were unable to collect meltwater samples until the column was kept at temperatures consistently above 0°C . Meltwater fractions were collected in 160-mL sterile glass dilution bottles, but only selected samples were analyzed, depending upon the number of available samples that could be processed that day. After the last meltwater fraction was taken, one bottle of sterile buffered dilution water was poured into the PVC column, the water in the column was then swirled, and the rinse-water sample was then collected from the bottom of the column.

Second indoor study

Prior to starting this experiment, the snow used in this study was moved from storage at -10°C (14°F) to -15°C (5°F), where it was stored for several weeks. The temperature of the incubator was cycled from temperatures that were well below freezing to slightly below freezing nine times ($\sim -7^{\circ}\text{C}$ to -1°C), from freezing to thawing temperatures once but held for four days ($\sim 0^{\circ}\text{C}$ to 5°C), and from temperatures slightly above thawing to warmer temperatures 12 times ($\sim -3^{\circ}\text{C}$ to 9°C). The complete cycling schedule is given in Table A2. The meltwater fractions were collected in 480-mL, sterile glass bottles. By collecting the samples in larger bottles, we were able to analyze all the meltwater fractions. The column was rinsed as described previously and the rinse water was collected.

Outdoor study

The snow used in this study was stored at -10°C . The snow column was placed outside in an open field on the morning of a sampling day. Meltwater fractions were drawn off until late afternoon. The column was then either left outdoors and sampled the next morning at approximately 7:00 a.m. or was placed in the environmental chamber at $\sim 0^{\circ}\text{C}$ for storage until the next sampling day. The schedule is given in more detail in Table A3. The meltwater fractions were collected as described in the previous study, and all fractions were analyzed. The column was rinsed as described previously and the rinse water was collected.

Microbiological analyses

Microbiological tests included enumerating the total number of heterotrophic bacteria at 37°C (the growth

temperature for human pathogens), total coliforms, fecal coliforms, and fecal streptococci. The tests for coliform bacteria and fecal streptococci are typically used to demonstrate contamination by feces, i.e., the presence of sewage bacteria. All the procedures used are given by the American Public Health Association (APHA), American Water Works Association (AWWA), and Water Environment Federation (WEF) (1992). The total Heterotrophic Plate Count (HPC) was prepared by using a Pour Plate Method with R2A agar. The membrane filter method was used for both the total coliform and fecal coliform tests. For the total coliform tests, M-Endo agar was used with verification of 10% of the colonies in Lauryl Tryptose broth followed by confirmation in Brilliant Green Lactose broth. The fecal coliform procedure used M-FC medium with agar added. Fecal streptococci were determined using the Multiple Tube Fermentation Technique with Azide Dextrose broth. All turbid tubes were streaked onto Pfizer Selective Enterococcus agar for confirmation.

RESULTS AND DISCUSSION

Snowmaking study

We were unable to detect either total coliform or fecal coliform bacteria after snowmaking. This was equivalent to more than a 3-log reduction in the total coliform counts and more than a 2-log reduction in the fecal coliform counts. The snowmaking process may be able to yield even larger log reductions but we were limited in our ability to detect any greater effect by the initial number of these bacteria present in the wastewater. The fecal streptococci were not nearly as adversely affected, with less than a 1-log reduction, or 72% loss (Table 2). The decrease in the total number of heterotrophic bacteria was almost 2 logs, from 2.0×10^5 CFU/mL to 2.2×10^3 CFU/mL (Table 2). Apparently, the gram-positive fecal streptococci survived this process better than the gram-negative coliforms. This agrees with findings on the effects of freezing and fro-

zen storage on these bacteria by McCarron (1965), Mackay (1984), and Kraft (1992).

Snow column studies

First indoor study

Figure 1 shows the color change in the first meltwater fractions. Table 3 gives information on the odor and color of the various meltwater fractions. Clearly, the first few bottles had the deepest yellow color and strongest odor. Figure 2 and Table 3 also show that there was another increase in color and odor on May 8. On May 19, there was a similar but less dramatic increase in odor and color (Table 3). Generally, later meltwater appears to be cleaner (Table 3). Thus, the contaminants responsible for color and odor are concentrated in the early runoff. The concentration phenomenon occurs several times during the course of this study; each time it is a little less pronounced than the previous event, and this concentration phenomenon appears to be associated with freezing followed by prolonged warming.

Table 3 also gives the mean total number of heterotrophic bacteria and mean number of fecal streptococci found in the snow and meltwater fractions. Although not shown in this table, we did not detect any total coliform or fecal coliform bacteria in any of these samples. When compared with the manufactured snow samples, the total number of bacteria was reduced in the initial meltwater samples but was approximately three orders of magnitude greater in the final meltwater samples (Table 3). The total number of bacteria in the last meltwater fraction (alone) was greater than the total number of bacteria in the whole snow column (Table 4). Therefore, we believe that the microbial growth occurred during the warming periods and was most pronounced when the "nighttime" or storage temperature was above 0°C. Some bacteria are able to grow at temperatures around 0°C. It is possible that a biofilm layer, rich in nutrients, formed at the bottom of the column and that this allowed growth of some species of bacteria. Because this environment would be rich in

Table 2. Effect of snowmaking on bacterial counts.

Sample	Mean total count (CFU*)	Mean total coliform (CFU)	Mean fecal coliform (CFU)	Mean fecal streptococci (CFU)
Wastewater	2×10^5 /mL	3.2×10^3 /mL	390/mL	2.2×10^4 /100-mL (or 220/mL)
Fresh snow	2.2×10^3 /mL	<1/10-mL (or <0.1/mL)	<1/10-mL (or <0.1/mL)	4.8×10^3 /100-mL (or 48/mL)
*Colony Forming Unit				



Figure 1. Color changes in first eleven meltwater samples taken from first snow column.

solutes, it would also be more likely to remain unfrozen even at temperatures slightly below 0°C.

The fecal streptococci were reduced by one to three orders of magnitude but were detectable throughout the experiment (Table 3). Unlike the total counts that increased throughout the experiment, concentrations of these microorganisms decreased slightly.

Second indoor study

To confirm our previous findings, we repeated the previous study with a few modifications. We did not perform coliform analyses because we had been unable to detect any coliform bacteria in either the snow or the meltwater fractions in the first study. Also, all the melted fractions were analyzed in this study.

Table 3. Results from first indoor study.

Sample	Sample date	Sample time	Fraction no.	Comments	Mean total count (CFU*/mL)	Mean fecal streptococci (CFU/100 mL)
Snow					2.7×10^3	3×10^3
Melted fraction	4/29	0900	1	yellow, bad smell	7.4×10^1	50
		0900	4	lt. yellow, bad smell	2.4×10^1	30
		0900	8	relatively clear	1.8×10^1	30
		0900	16	relatively clear	3.0	80
	5/7	0900	24	relatively clear	1.4×10^1	50
	5/8	0900	25	yellow, off odor	3.6×10^5	240
		0900	26	almost clear	4.8×10^4	23
		0900	30	lots of black specks	3.5×10^3	50
		1500	31	lots of black specks	2.2×10^4	30
	5/19	0700	32	slightly yellow	4.4×10^4	13
	5/20	0900	34	clear	2.9×10^4	4
	5/21	0830	36	clear	3.8×10^4	13
		1330	39	clear	2.9×10^5	13
	5/28	0845	40	clear with black specks	2.7×10^6	<2
		0845	41	clear	2.4×10^6	4
		0845	42	clear	2.4×10^6	2
		1300	43	clear	3.8×10^6	4
	5/29	0845	44	few black specks	2.2×10^6	2
		0845	45	more black specks	2.4×10^6	23
		0845	46	lots of black specks	6.2×10^6	23
		0900	Rinse		8.7×10^6	23

*Colony Forming Unit



Figure 2. Color change in meltwater fractions 25 through 30.

Table 5 gives descriptive information, total plate counts, and fecal streptococcal counts for each meltwater fraction. Again, the initial samples had significant color and odor while the final samples were relatively clear and had minimal odor. In this case we did not see the multiple concentration events that were observed in the first study, especially with respect to color. In the previous study, the concentration events were associated with a period of prolonged warmth that followed a significant freezing event. In this study, this occurred on August 4 and we observed that the first samples drawn after this event (on 8/6) had more odor than the previous samples.

Total counts were approximately 10^1 CFU/mL in the initial meltwater and increased to 10^6 CFU/mL by the end of the study when significant warming had occurred. Again, there was a significant increase in the number of bacteria in the meltwater when compared with the initial concentration in the snow sample (Table 4). Fecal streptococcal counts were generally <2 CFU/

100 mL, except for the final sample and rinse-water sample where the counts were only slightly higher, 2–4 CFU/100 mL. There does not appear to be any preferential survival of bacteria based upon gram reaction or cell wall composition, as approximately half (46% [11 of 24]) of the bacteria in the meltwater were gram positive (Table 5) and the remainder were gram negative.

Outdoor study

In this study, the snow column was placed outside, and all the fractions were collected and sampled. Table 6 gives information on the color and odor of these samples. We see a similar concentration effect in that the initial samples had significant color and odor. Samples drawn on 7/23 show a slight concentration event, corresponding with significant warming after a freezing event. The final samples had some sediment and thus were a little cloudy but had only a faint musty odor. Total counts fluctuated from 10^1 CFU/mL to 10^3 CFU/mL throughout the experiment and did not in-

Table 4. Summary of total numbers of bacteria in snow and meltwater-calculated values.

Study #	Total vol. meltwater in column ^a (mL)	Total number of bacteria in snow column ^b (CFU ^c)	Number of bacteria in last meltwater fraction ^d (CFU)	Total number of bacteria in all meltwater fractions ^e (CFU)
1	7360	2.0×10^7	9.9×10^8	—
2	7200	1.9×10^7	6.7×10^8	—
3	5760	1.73×10^6	7.2×10^5	2.3×10^6

^aTotal volume in column = (mL/bottle)(# bottles collected from column).

^bTotal number bacteria in snow = (# CFU/mL in snow)(total # mL melted snow in column).

^cColony Forming Unit

^dNumber bacteria in last meltwater sample = (# CFU/mL in sample)(# mL in sample).

^eTotal number bacteria in all the meltwater = Σ #5 for all the fractions.

Table 5. Results from second indoor study.

<i>Sample date</i>	<i>Sample time</i>	<i>Fraction no.</i>	<i>Description</i>	<i>Total count (CFU*/mL)</i>	<i>Fecal streptococci (CFU/100 mL)</i>
7/21	0930	1	yellow, smells bad	5.5×10^1	<2
7/27	1500	2	yellow, bad smell	3.4×10^1	<2
7/27	1500	3	relatively clear, some fine sediment, odor	3.1×10^1	<2
7/28	1045	4	relatively clear, musty odor	1.8×10^2	<2
7/28	1045	5	relatively clear, faint musty odor	3.0×10^2	<2
7/28	1500	6	relatively clear, faint scent	4.8×10^2	<2
7/29	0930	7	relatively clear, faint odor	5.3×10^2	<2
7/29	0930	8	relatively clear, faint odor	3.1×10^2	<2
7/30	0845	9	large sediment pieces	1.8×10^2	<2
7/31	1430	10	some large sediment, no distinct odor	4.9×10^2	<2
8/6	1445	11	some large sediment, musty odor	8.4×10^2	<2
8/7	1440	12	some medium-sized sediment, musty scent	7.8×10^2	<2
8/12	1500	13	a few medium sediment pieces, slight scent	1.3×10^4	<2
8/12	1530	14	some fine sediment, musty scent	$\sim 10^6$	<2
8/14†	0800	15	medium and fine sediment, musty odor	1.4×10^6	2
8/14	0830	Rinse water	Fine, medium, and large sediment, foul musty odor	1.2×10^6	4

*Colony Forming Unit
†The final sample was not collected until 8/14 as a large chunk of ice remained, and it took two days to melt.

crease dramatically as they did in the two previous studies. There was little or no increase in the total number of bacteria when the sum of the bacteria in each of the meltwater fractions is compared with the total number of bacteria in the snow column (Table 4). One reason there was no increase in bacterial numbers may be because of the relatively short time period for this experiment, three days vs. many weeks for the other studies. However, the total counts did not show a significant decrease, either. Thus it appears that sunlight did not have a strong negative impact on bacterial numbers. This finding is supported by literature reports that claim that the ultraviolet short wavelengths, which are ex-

tremely lethal to microorganisms, do not penetrate the earth's atmosphere (Brock 1970). Again, we see an almost identical survival rate of gram-positive and gram-negative bacteria in the meltwater, as 44% (12 of 27) of the bacteria were gram positive (Table 6).

DISCUSSION

With respect to the snowmaking process, our findings agree well with the unpublished findings of the Ontario Ministry of Environment (1982, as given by Zapf-Gilje 1985) in that the losses of total and fecal

Table 6. Results from third outdoor study.

Snow sample	Total coliform/ 100 mL	Fecal coliform/ 100 mL	Total count (CFU*/mL)	Fecal streptococci (CFU/100 mL)	
Snow	0	0	3×10^2	170	
Melted fractions					
Sample date	Sample time	Fraction no.	Description	Total count (CFU/mL)	Fecal streptococci (CFU/100 mL)
7/21	0930	1	yellow, bad smell	5.1×10^2	11
7/21	1035	2	yellow, smells	1.1×10^3	2
7/21	1440	3	pale yellow tint, slight odor	7.2×10^2	2
7/21	1440	4	pale yellow tint, slight odor	7.4×10^1	2
7/22	0820	5	cloudy, slight odor, quite a lot of fine sediment	4.8×10^2	<2
7/22	1215	6	cloudy, not yellow, faint musty odor, less sediment than #5	1.5×10^2	<2
7/22	1215	7	cloudy, not yellow, faint musty odor, less sediment than #5	6.0×10^1	2
7/23	0900	8	pale cloudy yellow, barely noticeable odor	1.5×10^2	2
7/23	0900	9	pale cloudy yellow, barely noticeable odor	1.0×10^2	<2
7/24	1020	10	some sediment, cloudy, cannot detect odor	8.2×10^1	<2
7/24	1500	11	some sediment, faint, barely noticeable odor	1.7×10^2	2
7/27	0830	12	sediment, faint musty odor	1.5×10^3	<2
7/27	0845	Rinse water	thick sediment, musty odor	6.6×10^2	—
*Colony Forming Unit					

coliforms were greater than two orders of magnitude in the snow as compared to the water going into the snow guns. Our results also agree with those of McCarron (1965) in that other species of bacteria were more resistant to freezing than *E. coli*. For the concentrations of total coliforms and fecal coliforms found in the initial wastewater used in this study, this treatment was effective in bringing coliform and fecal coliform levels below the regulatory levels required for most water quality discharge permits. However, if the total coliform or fecal coliform levels had been greater by a factor of 10 or 100, we do not know whether the treated water would meet water quality standards. Testing wastewater with higher concentrations of these bacteria would answer this question.

We found that many species of bacteria appear to be able to withstand the multiple freeze–thaw cycling that occurs in the snow column and replicate during the melting process. This may be because bacteria collect at the bottom of the snow column and the initial meltwater they receive is very nutrient-rich, allowing for their growth. The solutes in the meltwater would also help reduce the amount of freezing that might occur during this period. Many of the bacteria that survive the “winter” are gram negative, and may be organisms that are responsible for gastrointestinal illnesses.

Because the *E. coli* are very susceptible to this treatment process, it may be that fecal coliforms are not the best organisms to use as a measure of treatment efficiency for this type of treatment process. The Joint Task

Force of the American Society of Civil Engineers and the Water Environment Federation (1991) state that in the future, regulators may move toward use of other organisms as monitoring indicators. Ray (1989) noted that the ability of some pathogens (*Yersinia enterocolitica*, *Listeria monocytogenes*, and *Aeromonas hydrophilia*) to grow at refrigeration temperature and the susceptibility of fecal coliforms and *E. coli* to low temperature should be considered in determining their suitability as indicator bacteria.

There also is evidence that the standard enumeration method used for total coliforms, fecal coliforms, and fecal streptococci may result in erroneously low numbers because of the increased sensitivity of these bacteria when injured to many of the same compounds that are used in the selective media for their determination (Ray 1989). Thus, any future determination of the effectiveness of this type of treatment process should utilize microbiological methods designed to recover stressed organisms.

CONCLUSIONS

The purpose of this research was to examine the impact of the Snowfluent process on bacterial survival. Our literature review found that chilling, freezing, frozen storage, and warming all have a negative impact on bacteria, but that some species are much more seriously affected than others. Substrate, freezing and thawing rates, holding times, and bacterial age also greatly affect survival.

Our experimental studies specifically examined whether bacteria would survive snowmaking and the freeze-thaw processes that occur in snow during winter and the spring melt. We found that bacteria, which are capable of growing at the temperature of the human body and thus could be pathogens, survived the snowmaking process. Gram-negative coliforms were the most negatively affected by this process, with losses of two and three orders of magnitude for fecal coliforms and total coliforms, respectively. Fecal streptococci were less adversely affected, with a loss of less than one order of magnitude (72%). Both gram-positive and gram-negative bacteria survived the multiple freeze-thaw cycles in the snow columns and replicated during the melting process.

Given these findings, those of Sanin et al. (1994) for other types of microorganisms in frozen sludge, and those of Ridgway (1984) for other water treatment processes, it is possible that helminth eggs and viruses could also survive this treatment. Clearly, additional study is needed on the effect of this treatment process on other types of pathogens and what species or types of bacteria

are likely to survive this treatment. Future study should utilize methods that allow for enumeration of injured cells, especially when working with indicator microorganisms.

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APPENDIX A: DATA

Table A1. Temperature cycling schedule for first indoor study.

<i>Date</i>	<i>Time</i>	<i>Setting (°C)</i>	<i>Final reading at that setting (°C)</i>	<i>Time</i>	<i>Setting (°C)</i>	<i>Final reading at that setting (°C)</i>	<i>Time</i>	<i>Setting (°C)</i>	<i>Final reading at that setting (°C)</i>	<i>Time</i>	<i>Setting (°C)</i>	<i>Final reading at that setting (°C)</i>
Initial			-7									
3/27	1000	-2		1400	-7							
3/30	1100	-2		1600	-7							
3/31	0900	-2		1615	-7							
4/1	0915	-2		1710	-7	-4						
4/6	0900	-2	0	1630	-7							
4/7	0900	-1		1630	-6							
4/8	0900	-10		1245	-6							
4/10	0900	0		1530	-6							
4/13	0845	+2	+1	1430	-7							
4/15	0745	+6	+6	1600	-7	-4						
4/16	0830	+5	+5	1615	-5	-3						
4/17	0945	+5	+4	1530	-4							
4/20	0920	+5	+4	1600	-4	-2						
4/21	0830	+8	+6	1600	-3	-1						
4/22	0840	+9	+6	1140	-3	-3						
4/27	0715	+12	+8	1015	+13	+11	1500	+3				
4/28	0625	+12	+10	1500	+6							
4/29	0615	+12	+10	1345	+6		1530	0		1900	-2	+1
5/7	0615	-10		1055	+10		1310	+2				
5/8	0610	+10		1300	+2					1500	-5	
5/14	1330	-2		1515	+2							
5/15	0630	+10	+5	0930	+12	+9	1315	-3	-4			
5/18	0845	+10		1310	+5	+5	1500	+4	+5			
5/19	0700	+12	+10	1000	+11	+9	1600	+9	+7			
5/20	0900	+11	+8									
5/21	0600	+16	+15	1415	+1	+2						
5/25	*	+1	+2									
5/26	1400	+3	+6									
5/28	0745	+16	+14	1100	+18		1430	+11		1545	+10	+9
5/29	0605	+16	+14	0845	+18							

*No change in setting

Table A2. Cycling schedule for incubator in second indoor study.

<i>Date</i>	<i>Time</i>	<i>Setting (°C)</i>	<i>Final reading at that setting (°C)</i>	<i>Time</i>	<i>Setting (°C)</i>	<i>Final reading at that setting (°C)</i>	<i>Time</i>	<i>Setting (°C)</i>	<i>Final reading at that setting (°C)</i>
Initial			−4						
6/5/98	1030	−10	−9						
6/29	1100	−3	−2	1630	−10	−6			
6/30	0635	−3	−2	1630	−10	−6			
7/1	0735	−3							
7/2	1600	−10	−6						
7/6	0630	−3	−2	1600	−10	−7			
7/7	0600	−3	−1	1600	−10	−7			
7/8	0620	−3	−1						
7/9	1600	−10	−7						
7/10	0535	−3	−1	1200	−8	−6			
7/13	0900	−3	−1	1630	−8	−6			
7/14	0730	−3	−1	1540	−9	−6			
7/15	0700	−3	−1						
7/20	0900	+2	+1	1420	+3	+4			
7/21	0700	+8	+6	1500	+2	+2			
7/22	1415	0	+2						
7/27	1000	+8	+8	1445	+2	+3			
7/29	0610	+8	+8	1450	+2	+2			
7/30	0700	+8	+5.5	1650	+4	+2			
7/31	0650	+8	+11	1400	−2	−2			
8/4	1100	+2		1545	+6	+4			
8/5	0750	+8	+4	0910	+9	+5	1620	+6	+4
8/6	0810	+8	+4	0930	+10	+8			
8/7	0940	+12	+8	1600	+6	+3			
8/10	0810	+12	+7.5	1625	(+12)	+8			
8/11	0840	+14	+10	1230	(+14)	+9			
8/12	0830	+16	+10	1220	+10.5		1630	+18	

Settings in parentheses were not changed from previous setting.

Table A3. Cycling schedule for snow column in outdoor study.

<i>Date</i>	<i>Time</i>	<i>Action</i>	<i>Temperature range*</i>
7/21	0830	Column moved outdoors	18.9°C–31.1°C
7/22	1500	Column moved to incubator, set at 0°C	
7/23	0815	Column placed outside	18.9°C –20.6°C
	0845	Column moved to incubator because of impending rain, set at +2°C	
7/24	0820	Column placed outside	21.1°C–23.3°C
	1510	Column placed in incubator, set at +2°C	
7/27	0830	Column placed outside and last sample drawn	15°C–22.8°C

*Temperature range during time of outdoor exposure, based upon hourly readings at Lebanon Municipal Airport.

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